Effects of Seven Consecutive Years of Porcine Zona Pellucida Contraception on Ovarian Function in Feral Mares[1]

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ABSTRACT

A seven-year study was conducted to determine the effects of long-term treatment of wild mares with a porcine zona pellucida (PZP) immunocontraceptive vaccine on fertility, urinary estrogen and progesterone metabolite concentrations, ovulation rates, and reversibility of contraceptive effects. Fifty free-roaming mares were inoculated remotely with PZP in March over 7 consecutive years (1988–1994). During the initial year of treatment, each mare received either one (n = 14), two (n = 18), or three (n = 18) inoculations (2–3 wk apart) of 65 μg of native PZP; each received a single annual booster inoculation thereafter. Alternate-day urine samples were collected directly from the sand after witnessed eliminations. Collections covered 60 days during the peak of the breeding season. Ovarian endocrine function was monitored by means of urinary estrone conjugates (E_1C) and nonspecific progesterone metabolites (iPdG). PZP treatment over 6 yr, equaling 105 mare-years, resulted in four foals (3.8%) compared to a 46.2% foaling rate for the untreated population. Twenty-seven mares received their initial inoculations during the later stages of pregnancies (280–340 days), and all 27 delivered foals. Twenty-one of these foals lived to 1 yr, and this survival rate (77.7%) did not differ significantly from that of 25 foals (84% survival rate) born to untreated mares. As evidenced by pregnancies or luteal phase iPdG patterns and concentrations, ovulation rates declined over 6 consecutive years of treatment. The ovulation rates after a single year of treatment, 3 consecutive years, and 7 consecutive years were 73.3% (11 of 15), 55.5% (5 of 9), and 10% (1 of 10), respectively. One mare was withdrawn from treatment after 5 consecutive years of treatment and ovulated during the sixth and seventh years of the study.

After a single year of treatment, 12 of 15 mares (80%) had urinary E_1C concentrations that fell within the normal range of 100–385 ng/mg creatinine (Cr). After 2, 3, 6, and 7 yr of consecutive treatment, the percentage of mares with normal urinary estrogen concentrations declined to 46.1% (6 of 13), 33% (2 of 6), 40% (2 of 5), and 0% (0 of 5), respectively. Some mares with decreased urinary E_1C concentrations continued to demonstrate cyclic peaks and nadirs, suggesting continued but diminished follicular activity. Reproductive behaviors were correlated with elevated urinary E_1C concentrations. Reversibility of contraceptive effects after 1, 2, 3, and 4 consecutive years of treatment was 70% (7 of 10), 66% (2 of 3), 10% (1 of 10), and 50% (1 of 2), respectively. The results of this study indicate that long-term PZP treatment leads to a decline in both ovarian estrogen production and ovulation rates, and that reversibility of effects occurs in some animals.

INTRODUCTION

Immunocontraception of domestic and wild horses with a native porcine zona pellucida (PZP) vaccine has been shown to be an effective and safe method for inhibiting fertility [Liu et al., 1989; Kirkpatrick et al., 1990a, 1991; Kirkpatrick & Turner, 1991, 1992]. Contraceptive efficacy was shown to be greater than 95% after 2 yr of treatment [Kirkpatrick et al., 1991]. The vaccine can be delivered remotely via barbless darts, is safe to administer to pregnant mares, and will not affect the progress of the pregnancy or the health of the offspring [Kirkpatrick et al., 1990a]; and a single annual booster inoculation is sufficient to extend the contraceptive effects of the vaccine for subsequent years. The application of the PZP vaccine already has been extended to feral donkeys in Virgin Islands National Park, free-roaming wild horses in Nevada, and cap-

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tive exotic equids such as Przewalski's horse, zebras, and onagers [Kirkpatrick et al., 1992a].

Concern about the use of the PZP vaccine in wild and feral equids as well as in other species exists because of the possibility of long-term effects after many years of continuous treatment. Few studies have examined reversibility of contraceptive effects, ovarian endocrine function, or ovulation in animals treated over 3–7 yr. Among several species, even short-term treatment with a native PZP or the highly purified epitope ZP3 has led to disruption of both ovarian function and cyclic endocrine patterns. In dogs, PZP treatment of bitches resulted in long-term alterations in estrous cycles, a decline in ovarian estrogen secretion, and a depletion of ovarian follicles [Mahi-Brown et al., 1985]. In rabbits, PZP treatment has resulted in ovarian dysfunction, including failure to ovulate and lowered estrogen secretion [Wood et al., 1981; Skinner et al., 1983]. Dunbar et al. [1989] inoculated ten baboons with ZP3, and the results included reduced estrogen concentrations and a decline in the number of antral follicles. Two baboons failed to ovulate and five became amenorrheic by 8 mo after treatment.

Among domestic horses, a single year's treatment with PZP did not interfere with ovulation or reversibility of the contraceptive effects [Liu et al., 1989]. Among free-roaming wild horses receiving a single PZP treatment, reversibility of the vaccine's contraceptive effects has been demonstrated after a year without treatment [Kirkpatrick et al., 1991]. However, after 3 consecutive years of treatment, free-roaming wild horses showed reduced ovulatory rates and declining estrogen concentrations [Kirkpatrick et al., 1992b].

The objectives of the present study were to 1) test the contraceptive effectiveness of a native PZP vaccine given over 7 consecutive years, 2) determine the reversibility of the vaccine's contraceptive effects, 3) determine whether or not mares continued to ovulate after long-term treatment, and 4) determine whether or not cyclic estrogen patterns were affected by long-term PZP treatment.

MATERIALS AND METHODS

Animals

Fifty sexually mature mares were treated with the PZP contraceptive vaccine during the course of this study. Another 33 mares were used as either sham-treated (n = 6) or untreated controls during the study. All mares were free-roaming and inhabited Assateague Island National Seashore, MD. They received no supplemental feeding or veterinary care of any kind. The ages and fertility records were known for 46 of 50 treated mares and for 26 of 33 controls. Ages ranged from 3 to 18 yr, and each mare possessed unique markings that were used for identification purposes.

PZP Immunization

The PZP vaccine was prepared from porcine ovaries as described by Liu et al. [1989] and stored frozen until used in the field. A 1.0-cc adjuvant-vaccine emulsion was prepared in the field and administered to the mares in the gluteal muscles by means of barbless, self-injecting darts, at ranges of 25 to 40 m. Between 1988 and 1991, darts were delivered with a Pax-Arms (Timaru, New Zealand) .569-caliber capture gun. Between 1992 and 1994, darts were delivered by a Pneudart (Williamsport, PA) .50-caliber capture gun. The vaccine dosage was always 65 μg of antigen (or approximately 5000 zonae). Freund's complete adjuvant (FCA) was always used for the initial treatment, and Freund's incomplete adjuvant (FIA) was used for all subsequent inoculations.

In 1988 (Year 1), 26 mares received either two (n = 8) or three (n = 18) inoculations, given at approximately Days 1, 14, and 42, during February, March, and April as described by Kirkpatrick et al. [1990a]. In March 1989, 14 of these 26 mares received a single booster inoculation [Kirkpatrick et al., 1991]. In March 1990, and again in 1991, 10 of these 14 received a single annual inoculation. In 1992, 6 of these 10 mares received their fifth consecutive annual treatment; and in March 1993 and 1994, 5 of these 6 received their sixth and seventh consecutive annual treatment. In 1992, 24 new PZP-treated mares were added to the study group; and in 1993 and 1994, 10 of these were given annual booster inoculations. Table 1 provides a history of treatment for the mares included in this study.

Each October following treatment, all mares were tested for pregnancy on the basis of urinary estrone conjugate (E_1C) concentrations as described by Kirkpatrick et al. [1988]. Remote pregnancy testing was required because National Seashore regulations prohibit the capture or handing of any horses and because this method is more humane and causes less stress for the horses. Pregnancy testing was necessary in order to be absolutely certain that the

TABLE 1. History of PZP treatment for all mares in the study.

Year of treatment	N	Treatment
1988	26	All mares vaccinated with either 2 or 3 PZP inoculations.
1989	14	14 of the 26 mares given booster inoculations (12 are not).
1990	10	10 of the 14 1989 booster mares given third annual vaccination (4 are not).
1991	10	All of the same 10 mares given fourth annual vaccination.
1992	30	6 of the 10 1991 booster mares revaccinated (4 are not); 24 new mares given first vaccination.
1993	15	5 of the 6 1992 mares revaccinated (1 is not); 10 of the 24 1992 first-shot mares revaccinated (12 are not).
1994	15	5 of the 5 1993 booster mares revaccinated (seventh inoculation); 10 of the 10 1992 first-shot mares revaccinated (second booster).

failure to produce a foal was a consequence of contraception and not because a neonatal death had gone undetected. Differences in the rates of pregnancy between treated and untreated mares were tested for significance by means of binomial probability distribution [Freedman et al., 1978].

Assessment of Ovarian Function

Urine samples were collected every other day from urine-soaked sand after witnessed eliminations, as described by Kirkpatrick at al. [1992b], between 1 May and 30 June, from 1990 through 1994. The urine samples were diluted and assayed for E_1C and nonspecific progesterone metabolites (iPdG) by microtiter enzyme immunoassay. The E_1C assay has been described in detail by Shideler et al. [1993]. The measurement of E_1C has been validated for the *Equidae* [Daels et al., 1991; Czekala et al., 1990; Monfort et al., 1991] and reflects plasma estrogen concentrations (anti-E_1C antibody R 522 provided by B.L. Lasley, University of California, Davis); the historic inter- and intraassay coefficients of variation are 14.5% (n = 17) and 4.22% (n = 19), respectively. The lower limit of sensitivity for this assay was 6.25 pg, and concentrations of serially diluted urine samples collected from soil have exhibited parallelism to the standard curve [Kirkpatrick et al., 1988]. Recovery of ^{3}H-labeled estrone sulfate from the soil has been demonstrated to be > 95% [Kirkpatrick et al., 1988]. The enzyme immunoassay for iPdG has been described and validated in the *Equidae* by Kirkpatrick et al. [1990b] and employs monoclonal antibody 1284-1 raised against 20∂-hydroxyprogesterone (provided by R. Chatterton, Northwestern University, Evanston, IL). The inter- and intraassay coefficients of variation were 17.9% (n = 6) and 2.08% (n = 11), respectively; the lower limit of sensitivity was 0.07 ng. To account for differences in urine concentrations, all urine samples were assayed for creatinine (Cr) by the microcolorimetric method of Taussky [1954], and steroid metabolite concentrations were converted to ng/mg Cr. Ovulation was determined to have occurred in a mare if 1) iPdG concentrations rose to between 100 and 400 ng/mgCr, and then declined over an approximate 15–25-day period and 2) this iPdG rise and fall was preceded by an E_1C peak that coincided with an iPdG nadir [Kirkpatrick et al., 1992b].

The selection of mares for urine collection and measurement of steroid metabolites from among all treated mares was based on accessibility of the animals for urine collection. The May/June timing of urine collections was based on peak breeding activity among horses in this population [Keiper & Houpt, 1984]. Sexual behavior of the mares was assessed by limited direct observation and included 1) raised tail when stallions attempted mounting, 2) eversion of the clitoris, 3) absence of biting or kicking by the mare during attempted or successful mountings, and 4) copulation, as described by Waring [1983]. The period for which each mare was observed each day was variable and ranged from 1 to 3 h, depending upon the time between sighting of the mare and her urination. Differences in mean urinary E_1C concentrations across the 60-day collection period and across discernable estrous cycles, between mares, were tested for significance by means of Student's *t*-test [Freedman et al., 1978].

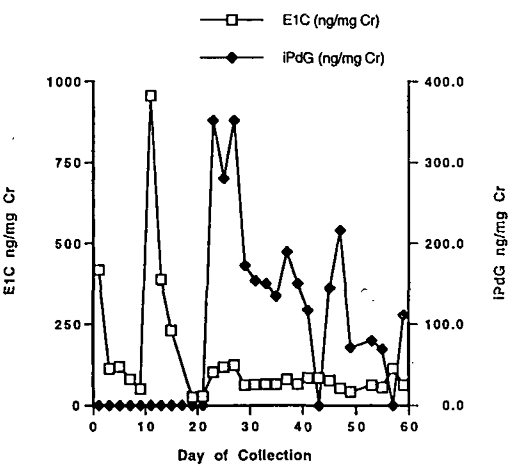

FIG. 1. Profiles of urinary E_1C and iPdG, indicating a nonconceptive estrous cycle in the untreated mare T3KO.

RESULTS

Porcine zona pellucida treatment over 6 yr, equaling 105 mare-years (the number of mares treated × the number of years treated) resulted in four foals (3.8%), compared to a 46.2% foaling rate for an untreated population for a period equaling 65 mare-years. The difference in foaling rate between PZP-treated and untreated mares was highly significant ($p < 0.001$). Twenty-seven mares received their initial PZP inoculations during the latter stages of pregnancy (280–340 days gestation), and all

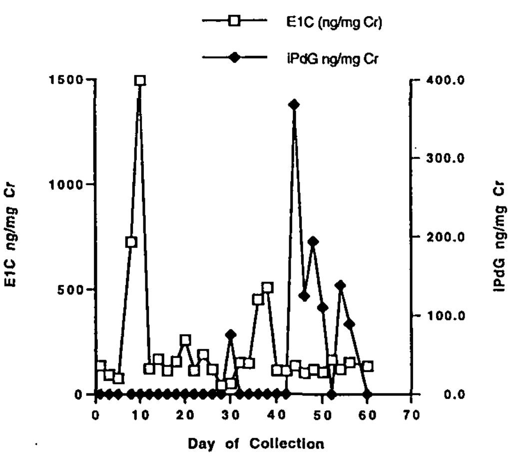

FIG. 2. Profiles of E_1C and iPdG in mare M61 during the sixth year of study. The preovulatory estrogen rise and the luteal phase iPdG pattern indicate ovulation after 5 consecutive years of PZP treatment.

27 delivered foals. Twenty-one of these foals survived for 1 yr (77.7%), and this degree of survival did not differ significantly ($p < 0.05$) from the survival of 25 foals born to untreated mares (84%) during the same period of time.

Reversibility of contraceptive effects was documented in 7 of 10 (70%) mares after a single year's PZP treatment, in 2 of 3 (66%) mares after 2-yr treatment, in 1 of 10 (10%) mares after 3-yr treatment, and in 1 of 2 (50%) mares after 4-yr treatment. Three mares that were in utero at the time their mothers were first inoculated, in 1988, have survived to maturity, and all three have produced foals of their own.

The ovulation rate after a single year of treatment was 73.3% (11 of 15) and after 2 yr of treatment was 60% (9 of 15). After 3 consecutive years of treatment the ovulation rate declined to 55.5% (5 of 9); and after 7 yr of treatment, the ovulation rate declined to 10% (1 of 10). Over the 7 yr, the ovulation rate for 4 control mares ranged from 75% to 100%. A typical luteal-phase ovulatory iPdG pattern is shown for the untreated mare T3KO (Fig. 1). One mare, M6I, was withdrawn from treatment after 5 consecutive years of PZP inoculations. She ovulated during Year 6 of the study but did not become pregnant (Fig. 2); she ovulated again during Year 7.

The normal range of urinary E_1C for cycling mares is 100–385 ng/mg Cr [Daels et al., 1991]. After a single year of treatment, 12 of 15 mares (80%) had urinary E_1C concentrations that fell within this normal range. After 2 yr of treatment, the percentage of mares with normal urinary estrogen concentrations declined (6 of 13) to 46.1%. After 3 consecutive years of treatment, 2 of 6 mares (33%) had normal urinary E_1C concentrations; after 6 yr of treatment, 2 of 5 mares (40%) had normal urinary E_1C concentrations, and after 7 yr of treatment, none of the 5 mares had normal urinary E_1C concentrations. By the seventh year of treatment, the difference between urinary E_1C concentrations in the 5 treated (14.21 ± 0.87 ng/mg Cr) and 4 control mares (246.88 ± 35.53 ng/mg Cr) was highly significant ($p < 0.0001$) during the May/June collection period. All reproductive behaviors that were observed were correlated with elevated urinary E_1C concentrations. About 50% of treated mares continued to demonstrate cyclic patterns of urinary E_1C. Of 16 mares, 7 showed decreased urinary E_1C concentrations but continued to demonstrate cyclic peaks and nadirs, suggesting continued but diminished follicular activity. This typical

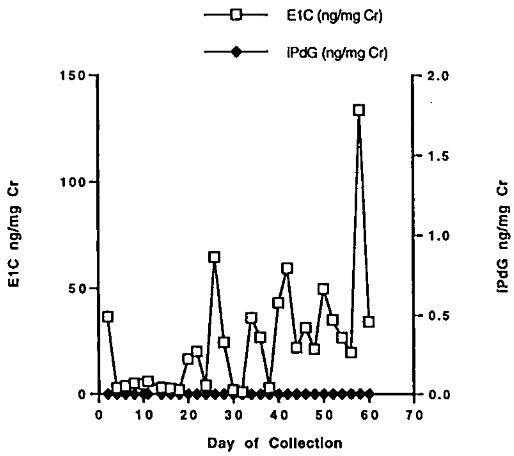

FIG. 3. Profiles of E_1C and iPdG for mare M2E after 6 consecutive years of PZP treatment. There are no indications of a luteal phase iPdG pattern or ovulation, but cyclic E_1C patterns suggest the partial maturation of follicular pools.

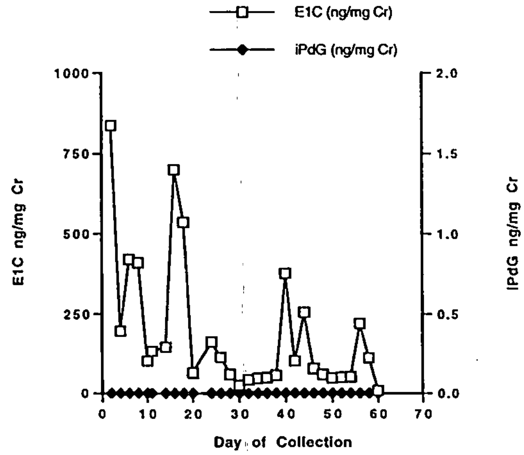

FIG. 4. Profiles of E_1C and iPdG for mare X1 after 7 consecutive years of PZP treatment. These profiles indicate cyclic estrogen waves approximately 20 days apart.

cyclic pattern, with diminished peaks approximately 20 days apart, is shown for mare M2E after 6 consecutive years of treatment (Fig. 3). The same type of urinary E_1C pattern is also seen in mare X1 (Fig. 4) after 7 consecutive years of treatment. However, M2E had urinary E_1C concentrations that were within the normal range. During Year 7 of the study, mare M6I, which was withdrawn from treatment after Year 5, had significantly higher ($p < 0.0001$) urinary E_1C concentrations (51.14 ng/mg Cr) than the 5 mares receiving 7 yr of treatment, but her urinary E_1C was still significantly lower ($p < 0.005$) than that of the 4 untreated controls.

DISCUSSION

Immunocontraception of free-roaming wild, feral and captive exotic equids with PZP is currently the most effective method of preventing unwanted births among these species. Contraceptive effectiveness exceeds 95%, and the small volume of the vaccine permits remote delivery to animals, thereby avoiding often expensive and stressful capture. However, the public's concern over the future of the wild horse, and the conservation value of rare captive equid species such as Przewalski's horse, are responsible for the requirement that any contraceptive approach be reversible. Thus, the possibility of long-term effects of PZP immunocontraception upon the ovary and the reversibility of contraceptive effects remain important issues.

The mechanism of action of PZP-induced contraception in mares is thought to be a block to fertilization [Liu et al., 1989]. One or more of the four major proteins of the noncellular zona pellucida appear to play a role as the receptor molecule for sperm surface molecules [Florman & Wassarman, 1985]. The role of the ZP3 molecule in the mare as a sperm receptor has been confirmed in vitro [Arns et al., 1991]. It appears that the acrosome reaction, which must precede fertilization, occurs with the attachment of the sperm surface receptors to the ZP3 molecule. Other glycoprotein components of the zona pellucida also may play a role in the fertilization process [Hasegawa et al., 1992].

Anti-PZP antibodies also may interfere with the development and maturation of ovarian oocytes in addition to blocking fertilization among ovulated ova. Henderson et al. [1988] have demonstrated that anti-zona antibodies will bind to the developing zona pellucida of the follicular oocytes. It is then possible that this binding with developing zonae disrupts some developmentally critical communication between follicular cells and the developing oocytes [Skinner et al., 1990]. This theoretical mechanism of action at the follicular level is consistent with the observed depletion of the oocyte pool in some species [Skinner et al., 1984].

The long-term effects of continuous PZP treatment have not been described in species

other than the horse. Reversibility of the contraceptive effect has been demonstrated in several species, but only after short-term application (< 3 yr) of the PZP vaccine [Gulyas et al., 1983; Sacco et al., 1987]. In the dog and rabbit, PZP immunization appears to be very damaging to ovarian follicles and often leads to cessation of ovarian function, with accompanying anovulation and depression of estrogen concentrations [Wood et al., 1981; Mahi-Brown et al., 1985]. Unusually large doses (5000 μg) have caused anovulation in the baboon [Dunbar et al., 1989].

These effects have not been demonstrated in the horse after short-term use, but there is evidence that some of these effects occur after 3 or more consecutive years of treatment. In the mare, treatment with PZP for a single year appears to lead to no disruption of ovarian function, and reversibility of contraceptive effects is complete. In domestic mares, Liu et al. [1989] demonstrated that the contraceptive effects of PZP treatment were reversible among 13 of 14 treated animals. Additionally, luteal phase plasma progesterone concentrations during the year immediately following contraception indicated that ovulation still occurred. Reversibility also appeared to be complete among a population of feral mares treated for a single year. Of 15 feral mares treated either two or three times in 1988, none produced foals in 1989, but 5 produced foals in 1990 [Kirkpatrick et al., 1991]. This is a normal foaling rate for these horses. Thus, it is clear that a single year's treatment of mares does not produce irreversible ovarian effects.

After 3 consecutive years of PZP treatment, 5 of 9 Assateague mares showed decreased urinary estrogen concentrations and no evidence of ovulation [Kirkpatrick et al., 1992b]; after 7 yr of treatment, only 1 of 10 mares showed evidence of ovulation, and several of these had depressed urinary estrogen concentrations. The evidence presented in the current study confirms the occurrence of long-term effects of PZP treatment in the mare.

On the basis of the information presented above, it is now necessary to determine the precise cause for long-term effects and to ascertain whether these long-term effects are permanent or transient. Despite the decline in ovarian function among mares treated for 3–7 yr, reversibility was documented in a small number of mares after 3 and 4 yr of PZP treatment. Also, one mare treated for 5 yr ovulated in the sixth and seventh year. Additionally, the presence of cyclic urinary estrogen peaks in some mares, approximating 21-day cycles, suggests that some follicles are still intact and are capable of maturation to some point short of ovulation. Several earlier studies with other species support the hypothesis that long-term effects can be transient [Gulyas et al., 1983; Sacco et al., 1983a,b; 1987; Bamezai et al., 1986].

Kirkpatrick et al. [1992b], speculating on the causes of the long-term effects in the mare, considered whether purity of the antigen and dose of antigen delivered determined the resulting concentration of antibodies. The purity of the antigen is an unlikely explanation for long-term effects in light of several other studies in which highly purified ZP3 or even epitopes of the ZP3 molecule were used but in which temporary ovarian dysfunction still occurred [Paterson & Aitkin, 1990; Dunbar et al., 1989; Sacco et al., 1987]. However, Hasegawa et al. [1992] immunized hamsters with either 20 μg of PZP or a purified ZP4. Infertility was complete and irreversible in those hamsters immunized with the PZP, but contraceptive effects were temporary in the animals immunized with ZP4. Histological examination of ovaries revealed a complete lack of follicles in the PZP-treated hamsters, while the animals treated with ZP4 had less damage to follicles. Thus, the issue of antigen purity is equivocal. Current studies, including the one reported here, have not allowed separation of the issues of antibody concentration and duration of dose—largely because of the differences between studies, with dosages ranging from 65 μg for 300-kg animals to 5000 μg for 30-kg animals. Thus, future studies must focus upon dose response and the degree and duration of ovarian dysfunction that result from treatment.

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